ECURITY CLASSIFICATION OF THIS PAGE

(2)

REPORT DOCUMENTATION PAGE

1a. **AD-A266 513**

2a.

2b.

1b. RESTRICTIVE MARKINGS

3 DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; distribution is unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
NMRI 93-40

5 MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION
Naval Medical Research Institute

6b. OFFICE SYMBOL (*If applicable*)

7a. NAME OF MONITORING ORGANIZATION
Naval Medical Command

6c. ADDRESS (*City, State, and ZIP Code*)
8901 Wisconsin Avenue
Bethesda, MD 20889-5055

7b. ADDRESS (*City, State, and ZIP Code*)
Department of the Navy
Washington, DC 20372-5120

8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research & Development Command

8b. OFFICE SYMBOL (*If applicable*)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (*City, State, and ZIP Code*)
8901 Wisconsin Avenue
Bethesda, MD 20889-5044

10 SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO
61102A 61102A	3M263002 3M161102	DB10AJ1294 BS13AK1291	DN243591 DN243531

11. TITLE (*Include Security Classification*)
Signifance of duplicated flagellin genes in campylobacter

12. PERSONAL AUTHOR(S)
Alm RA; Guerry P; Trust TJ

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (*Year, Month, Day*)	15. PAGE COUNT
journal article	FROM _____ TO _____	1993	5

16. SUPPLEMENTARY NOTATION
Reprinted from: Jounal of Molecular Biology 1993 Vol.230 pp. 359-363

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (*Continue on reverse if necessary and identify by block number*)
flagella; chimeric flagellin; campylobacter; recombination; flagellar motility

19. ABSTRACT (*Continue on reverse if necessary and identify by block number*)

93-15278

93 7 06 003

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS

21 ABSTRACT SECURITY CLASSIFICATION
Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL
Phyllis Blum, Librarian

22b. TELEPHONE (*Include Area Code*)
(301) 295-2188

22c. OFFICE SYMBOL
MRL/NMRI

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

J. Mol. Biol. (1993) 230, 359–363

COMMUNICATIONS

Significance of Duplicated Flagellin Genes in *Campylobacter*

Richard A. Alm[1], Patricia Guerry[2] and Trevor J. Trust[1]†

[1]*Department of Biochemistry and Microbiology*
University of Victoria, Victoria, British Columbia
V8W 3P6, Canada

[2]*Enteric Diseases Program, Naval Medical Research Institute*
Bethesda, MD 20814, U.S.A.

(Received 1 October 1992; accepted 11 December 1992)

The complex flagellum of *Campylobacter coli* VC167 contains two highly related (98%) flagellin subunit proteins which are produced from two 92% homologous, tandemly orientated genes, *flaA* and *flaB*. Mutants expressing only *flaA* form a full-length flagellar filament that confers slightly less than wild-type motility to the bacterium. However, flagellin mutants expressing only *flaB* produce extremely short, truncated filaments, and are only slightly motile. We have shown that the presence of two essentially identical genes is advantageous, in that *flaAflaB*+ mutants become highly motile upon passage by an event which allows the production of a full length simple flagellar filament containing a single FlaA-FlaB chimeric flagellin protein. Furthermore, we have demonstrated that the reassortment of DNA that results in this chimeric protein can occur by two mechanisms: intragenomic recombination and transformation-mediated intergenomic recombination.

Keywords: flagella; chimeric flagellin; *Campylobacter*; recombination; motility

Duplicated genes have been identified in a variety of eubacteria and archaebacteria. Such gene duplications may in fact be a mechanism by which bacteria can amplify particular functions, thus adapting to stressful or unfavourable conditions (Sonti & Roth, 1989). Indeed, multiple copies of genes for important virulence factors have been found in the genomes of pathogenic bacteria either in expressed or silent *loci* (Haas & Meyer, 1986; Mekalanos, 1983; Swanson *et al.*, 1986).

The thermophilic spiral organisms *Campylobacter coli* and *Campylobacter jejuni* are among the most frequently isolated enteric pathogens, causing severe diarrhea in humans (Butzler & Skirrow, 1979; Skirrow, 1977; Walker *et al.*, 1986). The polar flagellum of the *Campylobacter* cell imparts high motility to the bacterium and plays an important role in colonization of the viscous mucous lining of the gastrointestinal tract, and is regarded as an important virulence determinant (Black *et al.*, 1988; Caldwell *et al.*, 1985; Morooka *et al.*, 1985; Pavlovskis *et al.*, 1991). The flagellar filament of *C. coli* is complex. In the case of *C. coli* VC167, the flagellar filament is composed of two highly homologous 572 amino acid residue flagellin subunit proteins. These subunit flagellins are encoded by two tandemly orientated genes, *flaA* and *flaB* which display 92% identity within their coding regions (Guerry *et al.*, 1992). A second flagellin gene has been reported in *C. jejuni* strains 81-116 and IN1 (Fischer & Nachamkin, 1991; Nuijten *et al.*, 1990), and evidence now exists that all *Campylobacter* isolates carry duplicated flagellin genes, with significant homology (R. A. Alm, P. Guerry & T. J. Trust, unpublished results). Mutants that only express the *flaA* gene produce flagellar filaments that are indistinguishable in size from the wild-type strain and are composed solely of FlaA flagellin. Mutants that only express the *flaB* gene product produce a severely truncated filament that imparts only partial motility to the cell (Guerry *et al.*, 1991).

The conservation and stability of the tandemly duplicated flagellin genes in *Campylobacter* suggests that this property must endow these bacteria with a significant strategic advantage that outweighs the genetic instability of such an arrangement because tandem gene duplications are usually rapidly eliminated in bacteria by intragenomic recombination (Anderson & Roth, 1981; Tilsty *et al.*, 1984). Using the previously described *flaAflaB*+ mutant KX15 (Guerry *et al.*, 1991), we demonstrate here that the extra genetic information of a second highly homologous *fla* gene carried by *Campylobacter* strains can

† Author to whom all correspondence should be addressed.

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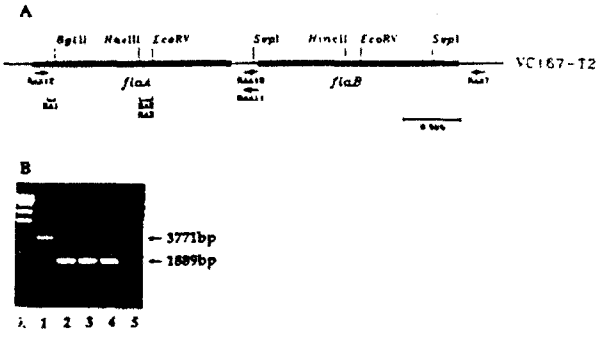

Figure 1. (A) Schematic representation of the flagellin genes, *flaA* and *flaB* of *Campylobacter coli* VC167-T2. The relative positions of the specific oligonucleotides used for PCR amplification are indicated. The unique restriction endonuclease sites within the flagellin genes used for determination of the recombinational cross-over points are shown. The areas in which recombination occurred to create the chimeric coding sequences for each of the mutants is designated. (B) PCR analysis of the wild-type VC167-T2 (lane 1), and representative chimeric mutants RA1 (lane 2), RA2 (lane 3) and RA3 (lane 4), and a no DNA control (lane 5). Lane 1 shows a 3·771 kb fragment representing 2 intact flagellin genes, whereas a 1·889 kb fragment is seen for the chimeric mutants in lanes 2, 3 and 4. Lambda (λ) size markers are bacteriophage lambda DNA digested with *Hin*dIII.

be used as a reserve copy to assure the organism of motility.

The *flaAflaB*+ mutant KX15 was constructed by insertion of a kanamycin (Km†) cassette into the unique *Eco*RV site of *flaA* (Fig. 1(A); Guerry *et al.*, 1991). We observed, after plating KX15 on Mueller-Hinton medium (BBL, Cockeysville, MD) containing 0·4% Bacto-agar (Difco), that highly motile bacteria irregularly swarmed out from the zone of growth after 24 to 36 hours' incubation at 37 °C. All of the 58 independently isolated motile colonies tested were Km^S. The flagellins from four of these motile cells were purified and subjected to automated Edman sequence analysis. In all cases, it was found that the first 27 N-terminal residues corresponded to that of the FlaA flagellin, GFRINTNVAALNAKANSDLN SRALDQS. We reasoned that, in order to become motile again, the *Campylobacter* cell had rearranged its flagellin information and was expressing a chimeric flagellin, consisting of both *flaA* and *flaB* coding regions. To examine this possibility, we used the oligonucleotides RAA12 (5′-CAGCAGAGCATTAGATCAATCACTTTCA-3′) that was specific for the N-terminal sequence of *flaA* beginning at position 63 and RAA7 (5′-ATCATGAAGAAAGTTTAATTGCCCCT-3′) which was specific for non-flagellin information 231 bp downstream from *flaB* (Fig. 1(A)) in a polymerase chain reaction (PCR). AmpliTaq polymerase (Perkin Elmer Cetus, Rexdale, Ontario) (0·4 Units/10 μl reaction) was used in a hot air thermocycler (Idaho Technology, Idaho Falls, Idaho) in the presence of 1 × standard PCR buffer and 2·5 mM $MgCl_2$ (GeneAmp, Perkin Elmer Cetus, Rexdale, Ontario) and a final concentration of 250 μM for each primer and 50 ng genomic template prepared as described previously (Alm, Guerry & Trust, unpublished results). Using the RAA12,RAA7 primer pair, and PCR cycle parameters of 1 second at 94 °C, 1 second at 55 °C and a 120 second extension at 74 °C for 35 cycles, it was possible to amplify a product of 1889 bases which correlates to a unit length flagellin gene from independently isolated mutants (Fig. 1(B)). Therefore, this represented a straightforward recombinational event between *flaA* and *flaB*, and in all these cases the Km^R cartridge and part of both flagellin genes had been deleted during the recombination. By restriction endonuclease digestion of the PCR product generated using the RAA12,RAA7 primer pair from these mutants with *Bgl*II, or *Hae*III, both of which are specific for the *flaA* gene, or *Hinc*II and *Ssp*I which are unique in the *flaB* sequence (Fig. 2), followed by separation on a 12% acrylamide gel according to the method described by Sambrook *et al.* (1989), we were able to determine the areas in which recombination had occurred to produce the chimeric coding sequence (Fig. 1(A)). The recombinational crossover in mutant RA1 was localized to the area between the 3′ end of primer RAA12 and the *Bgl*II site, a distance of 72 bp. The point of recombination in mutants RA2 and RA3 lay between the *Hae*III and *Eco*RV restriction sites, a distance of 97 bp. The high homology between the two *fla* genes does not enable the determination of the exact position of the recombinational event.

However, when the selective pressure for Km^R (100 μg/ml) was kept during growth of the *flaAflaB*+ mutant KX15 on the motility agar, spikes of the swarming growth caused by highly motile bacteria were still apparent, although the time for this growth to occur was increased to 60 to 72 hours. With the need to maintain the Km^R cartridge, the genetic rearrangements encountered were not always as simple as those described above. PCR and Southern analysis indicated that duplications of flagellin information had occurred, with extra partial copies containing *fla* gene information, comprising the 3′ end of the *flaA* gene (data not shown). When the *flaAflaB*+ mutant KX15 was fed to rabbits, the strain consistently failed to colonize and was cleared by 24 hours, providing further evidence that full motility is a crucial colonization determinant. No Km^S revertants were isolated from the rabbit, and the few Km^R colonies that were recovered were shown to display poor motility similar to the original *flaAflaB*+ mutant. However, after only two days' incubation on motility agar, one isolate displayed a phenotype of irregular motility identical to the Km^S mutants producing the chimeric flagellin, yet retained Km^R. Purification and N-terminal sequencing of the flagellin produced by this isolate demonstrated that it possessed the first 27 N-terminal residues identical to FlaA. PCR analysis of this mutant using primer pairs that

† Abbreviations used: Km, kanamycin; bp, base-pairs; PCR, polymerase chain reaction.

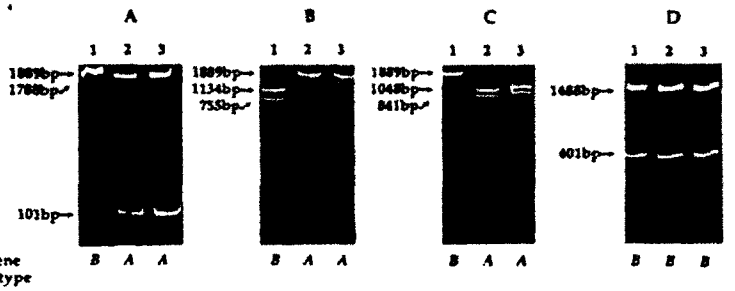

Figure 2. Restriction analysis of the 1·889 kb PCR product generated with RAA12 and RAA7 from mutants RA1 (lane 1), RA2 (lane 2) and RA3 (lane 3) using enzymes that have unique sites in *flaA* or *flaB*. *Bgl*II (panel A) and *Hae*III (panel C) are unique in the *flaA* gene while *Hinc*II (panel B) and *Ssp*I (panel D) are unique in *flaB*. The corresponding flagellin gene phenotype is shown under each lane, and size markers for the bands are indicated on the left of each panel.

could specifically amplify either the *flaA* (RAA12/RAA11: 5'-TGCATCGAAAAGATTAAAGCAAGA-3') or the *flaB* (RAA7 RAA10:5'-TCTTGCTTTAAT-CTTTTCGATGCA-3') region demonstrated that the Km^R cartridge was now located in the *flaB* gene, and the *flaA* gene was functional and intact (Fig. 3).

The rearrangement of flagellin information resulting in the restoration of full motility could be occurring by two distinct mechanisms, involving either intergenomic or intragenomic recombinational events. When the selective pressure for Km^R is removed, a simple recombination could occur between the two highly homologous flagellin genes. However, an event which would maintain Km^R would likely involve DNA liberated by cells that had undergone autolysis, and taken up via natural transformation. The requirement for cell death may be one explanation as to why a longer incubation time is required to observe Km^R motile revertants. To test this hypothesis, motility plates containing DNAse I (40 μg/ml) with and without Km were used. When the *flaAflaB*+ mutant was grown on motility plates containing DNAse without the selective pressure of Km, all 48 colonies were motile after 24 to 36 hours' growth, and were Km^S. Flagella were purified from three independent colonies, and in all cases upon N-terminal protein sequencing, the flagellins displayed the first 27 N-terminal residues of FlaA. PCR analysis in conjunction with restriction endonuclease digestion demonstrated that the gene encoding this flagellin was composed of both *flaA* and *flaB* sequences. However, when both Km and DNAse were added to the motility plate, the cells remained non-motile after 5 days' incubation at 37 °C. This confirms that the processes of natural transformation combined with intergenomic recombination were crucial for the rearrangement of flagellin information when the pressure of kanamycin was maintained. Although a mechanism similar to that described for the *Vibrio cholerae* RS1 element duplication is theoretically possible (Goldberg & Mekalanos, 1986; Mekalanos, 1983), we were unable to isolate any motile revertants in the presence of both DNAse and Km, indicating that natural transformation mediated recombination is the predominant mechanism.

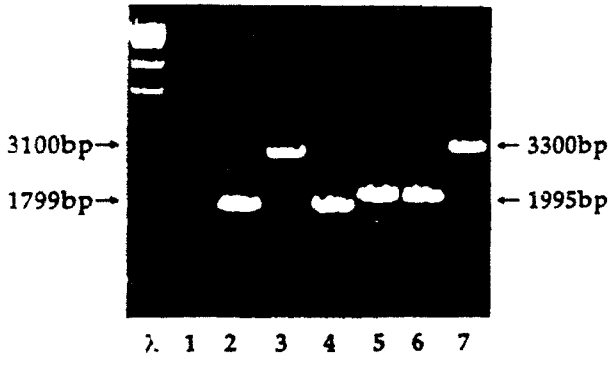

Figure 3. PCR analysis of VC167-T2 (lanes 2, 5), the original *flaA*−*flaB*+ mutant KX15 (lanes 3, 6) and the chimeric mutant isolated after animal passage (lanes 4, 7) and a no DNA control (lane 1). The specific oligonucleotide pairs used were RAA12/RAA11 (lanes 2 to 4) to amplify the *flaA* region, and RAA10/RAA7 (lanes 5 to 8) to amplify the *flaB* region. The increased size due to the presence of the 1·3 kb Km^R cartridge can be seen in the *flaA* gene of KX15 (lane 3), and the *flaB* gene of 663 (lane 7). Lambda (λ) size markers are lambda DNA digested with *Hin*dIII.

One would assume that there exists a functional reason why two flagellin genes are stably maintained in wild-type *Campylobacter* strains. The two *fla* genes are controlled by two different classes of promoters. The *flaA* gene is regulated by a σ^{28} promoter, similar to the flagella genes of *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis* (Guerry *et al.*, 1991; Helmann & Chamberlain, 1987), whereas the *flaB* gene is controlled by a σ^{54} promoter similar to the flagellin and hook genes of *Caulobacter* (Minnich & Newton, 1987; Mullin & Newton, 1989). FlaB is environmentally regulated and differentially expressed at different stages during the *Campylobacter* life cycle which allows the cell to modulate its motility (Alm, Guerry & Trust, unpublished results). The fact that the two *fla* genes are under the control of distinct classes of promoters, and are expressed at vastly different levels under standard growth conditions (Guerry *et al.*, 1991; Guerry *et al.*, 1990) may be vital to the stable maintenance of the tandem *fla* genes.

Moreover, the amino and carboxy terminal domains, normally highly conserved among eubacterial flagellins (Joys, 1985; Trachtenberg & DeRosier, 1988; Wei & Joys, 1985) are important for flagellin export, polymerization into a flagellum and filament stability (Fedorov *et al.*, 1988; Vonderviszt *et al.*, 1989, 1991). However, it is in these regions that 70% of the amino acid differences between the FlaA and FlaB flagellins of *C. coli* VC167 occur. These sequence differences may contribute to different structural roles for the two flagellins in the assembled filament, and these functional differences could also contribute to the stable maintenance of the duplicated genes in wild-type cells. Interestingly, we never isolated a mutant capable of producing a full length FlaB filament. The fact that all the full length filaments producing FlaA-FlaB chimeric flagellins we have isolated always contain the FlaA amino terminal domain suggests that if any structural constraint contributes to the inability of FlaB to produce a full length filament, this constraint must reside in the sequence differences carried in the N-terminal domain.

In summary, this study has provided additional evidence of the biological importance of tandemly duplicated flagellin genes in *Campylobacter*. Motility appears to be crucial to the ability of *Campylobacter* to colonize its intestinal niche, and the presence of a second flagellin gene can, under certain circumstances such as any possible spontaneous mutations which may impair motility, provide this organism with a backup or reserve gene copy, allowing the organism to maintain close to full motility. Moreover, two distinct and independent mechanisms can be utilized to accomplish this rearrangement of its genetic information, emphasizing the importance of motility to *Campylobacter*. Therefore, in addition to any structural and/or regulatory advantages in maintaining two highly homologous tandem *fla* genes (Guerry *et al.*, 1991; Alm, Guerry & Trust, unpublished results), the presence of duplicated flagellin genes and their ability to ensure motility appears to endow this pathogenic bacterium with a significant biological advantage.

This work was supported in part by a grant to T.J.T. from the Medical Research Council of Canada and to P.G. by U. S. Navy Research and Development Command. R.A.A. was the recipient of an NSERC International Postdoctoral Fellowship. Dr. O. Pavloskis is thanked for animal experiments.

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Edited by R. Huber